DNA CONTENT ANALYSIS FOR DETECTION OF APOPTOTIC CELLS

DNA content measurement with flow cytometry can be used to detect apoptotic cells with diminished DNA content. In this protocol, cells are fixed in ethanol before being subjected to mild extraction of low-molecular-weight DNA that leaks from the cells. Samples are then stained with PI in the presence of RNase A and analyzed by flow cytometry.

Analysis of apoptosis as presented in this protocol can be combined with electrophoretic analysis of low-molecular-weight DNA extracted from fixed cells by the phosphate-citrate buffer; for description of such a procedure for agarose gel electrophoresis, see Support Protocol. Combining the methods presented in this protocol and the Support Protocol enables one to simultaneously analyze the molecular weight of DNA extracted from the very same cells that are subjected to flow cytometry.

Materials

70% ethanol
Cells to be analyzed
Phosphate-buffered saline (PBS)
DNA extraction buffer: 0.2 M phosphate-citrate buffer, pH 7.8
Propidium iodide (PI) Triton X-100 staining solution with RNase A

15-ml polypropylene centrifuge tubes and caps
Flow cytometer with 488-nm argon ion laser fluorescence excitation source

Fix cells in ethanol

1. Distribute 10-ml aliquots of 70% ethanol into 15-ml polypropylene centrifuge tubes. Keep tubes in ice.

2. Suspend 1-5 x 10^6 cells in 1 ml PBS. Fix cells in suspension by rapidly admixing, with a Pasteur pipette, 1 ml cell suspension into 10 ml of 70% ethanol in centrifuge tubes on ice. Fix cells 2 hr.

Cells can be stored in fixative at –20 Deg C for several weeks.

Extract low-molecular-weight DNA from cells

3. Centrifuge cells 5 min at 200 x *g*. Thoroughly decant ethanol. Add 50 ul DNA extraction buffer. Transfer tubes to a 37 C water bath, cap, and incubate 30 min on the shaker.

The volume of DNA extraction buffer may vary. If the extracted DNA will be subjected to gel electrophoresis small volumes (~50 ul) should be used. This simplifies subsequent steps in the gel electrophoresis procedure: such small volumes may be directly incubated with RNase and proteinase K and loaded on the gel without the necessity of concentrating the DNA.

The efficiency of DNA extraction in step 3 should be controlled for optimal separation of apoptotic cells. If DNA degradation within apoptotic cells is extensive or if the cells have already shed apoptotic bodies, there is no need to extract low-molecular-weight DNA in step 3, as the apoptotic cells will already have a significantly reduced DNA content and will be well resolved on DNA content frequency histograms. On the other hand, if DNA degradation is incomplete and sub-G_1 and G_1 peaks are not separated, try extending the rinsing times (e.g., up to 2 hr) and using greater volumes of DNA extraction buffer (e.g., up to 500 ul).

4. Centrifuge cells 10 min at 1500 x *g*. Reserve supernatant for analysis of low-molecular-weight DNA by agarose gel electrophoresis.

Stain cells with PI

5. Resuspend cells in 1 ml PI staining solution with RNase A. Keep 30 min at room temperature, protected from light.

Perform flow cytometry

6. Set up and adjust flow cytometer for excitation with blue light and detection of PI fluorescence at red wavelengths.

For excitation, the 488-nm argon ion laser line may be used; a BG 12 optical filter is recommended when the source of illumination is a mercury arc or

xenon lamp. For detecting PI emission, a long-pass (600 nm) filter is recommended.

7. Measure cell fluorescence in a flow cytometer. Use the pulse width-pulse area signal to discriminate between G_2 cells and cell doublets and gate out the latter.

Note: Procedure adapted from the Protocols in Cytometry

Requirements for Submission of Flow Cytometry Samples

1. All samples must be submitted in 12X75 mm tubes (Falcon #2058 polystyrene with caps/NIH #6640-00-264-7731 or Falcon #2052 polystyrene without cap/NIH #6640-00-247-6372)

2. Cell concentrations should be a minimum of $0.5X10^6$ per ml and should not exceed $2.0X10^6$/ml-minimum sample volume 0.5ml and maximum volume 2.0ml.

3. All samples must be filtered through nylon mesh screen (Small Parts Inc. PO Box 4650, Miami Lakes, FL 33014-0650 1-800-220-4242/Part numbers R-CMN-62 (62 micron) or R-CMN-53 (53 micron) see www.smallparts.com for details. Sheets of mesh can be cut to 1"x1" pieces for filtering individual samples.

4. Each set of samples must be accompanied by the appropriate control specimens.

For immunofluorescence/phenotyping studies, unstained and isotype control specimens should be submitted to establish background/autofluorescence properties.